Characterisation of a new rubredoxin isolated from *Desulfovibrio desulfuricans* 27774: definition of a new family of rubredoxins

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Abstract A new rubredoxin from the sulphate-reducing bacterium *Desulfovibrio desulfuricans* ATCC 27774, grown with nitrate as terminal electron acceptor, was isolated and characterised. The protein is an 8.5 kDa monomer containing one iron atom per molecule, with a reduction potential of 25 ± 5 mV at pH 7.6. Like the recombinant Rdl protein from *D. vulgaris*, expressed in *Escherichia coli* [Lumpio, H.L., Shenvi, N.V., Garg, R.P., Summers, A.O. and Kurtz, D.M., J. Bacteriol. 179 (1997) 4607–4615], it contains an unusual spacing of four amino acids between the first two of the iron coordinating cysteinyl residues. This difference is reflected in the structure of the iron centre, as observed by visible and EPR spectroscopies. All together, these features make these proteins the first members of a new family of rubredoxins.

Key words: Rubredoxin; Rubrerythrin; *Desulfovibrio*; Electron paramagnetic resonance; Redox property

1. Introduction

Rubredoxins (Rds) are small, non-haem iron proteins, in which the metal is coordinated in a tetrahedral geometry to the sulphur atoms of four cysteinyl residues (for a recent review, see [1]). They are found in several anaerobic bacteria and, in particular, in all species of the sulphate-reducing bacteria belonging to the genus *Desulfovibrio* which have been studied so far. Their small molecular mass and their remarkable stability have made them choice proteins for X-ray crystallography as well as for metal substitution studies. For example, a nickel-substituted Rd has been shown to have hydrogenase activity [2].

The role of rubredoxins in sulphate-reducing bacteria is not yet clearly established with the exception of *D. gigas*, in which Rd has been shown to function as a redox coupling protein between NADH oxidoreductase and rubredoxin oxygen oxidoreductase [3,4]. These three proteins allow *D. gigas* to increase the production of ATP from the degradation of internal polyglucose, in the presence of oxygen [5]. This bacterium contains an even smaller protein, named desulforedoxin (Dx) [6] which also contains an FeS_4 centre [7]. In spite of the similarity between the Rd and Dx iron centres, Dx cannot replace Rd in transferring electrons from the *D. gigas* NADH oxidoreductase to the Rd oxygen oxidoreductase [3].

D. desulfuricans strain 27774, a bacterium capable of using either sulphate or nitrate as terminal electron acceptor, also contains a rubredoxin (from now on, named Rd-1), whose crystallographic structure has been determined with excellent resolution [8]. Two other interesting proteins containing mononuclear iron sites are found in this organism: one [9], first discovered in *D. vulgaris* [10], has been termed rubrerythrin and contains both a Rd-like centre and a binuclear iron centre [11,12]; the second, called desulfoferrodoxin (Dfx) [13], contains both a Dx centre and a new blue, square pyramidal $FeSN_4$ iron centre as revealed by the recent crystal structure determination of this protein [14,15]. Interestingly, *D. gigas* contains another non-haem iron protein, named neelaredoxin, which contains an iron site similar to the blue centre of Dfx [16].

Here we describe the purification and physico-chemical properties of a new rubredoxin (Rd-2) from *D. desulfuricans* grown on a medium with nitrate as terminal electron acceptor. The amino acid sequence of the protein is homologous to that whose gene has been found in *D. vulgaris*, an organism which is not capable of growth using nitrate as terminal electron acceptor. The recombinant *D. vulgaris* protein, which has been designated Rdl for rubredoxin-like protein, also contains the cys-X_4-cys motif found in the *D. desulfuricans* Rd-2 and therefore, these proteins constitute the first members of a new family of rubredoxins.

2. Materials and methods

2.1. Cell growth and protein purification

D. desulfuricans (ATCC 27774) was grown on a lactate/nitrate medium as described previously [17]. All purification steps, unless otherwise indicated, were performed in an anaerobic chamber (Coy model 1-2463) at 4°C and at pH 7.6. About 560 g wet weight cells were suspended in 600 ml of 10 mM Tris-HCl buffer and ruptured in a Manton-Gaulin press at 9000 psi. The bacterial extract was centrifuged at 19 000 × *g* for 30 min. The pellet was discarded and the supernatant subjected to further centrifugation at 180 000 × *g* for 30 min. The clear supernatant (700 ml) was applied on a DEAE-cellulose column (6 × 34 cm) previously equilibrated with 10 mM Tris-HCl buffer. Rd-2 was eluted at about 30 mM Tris-HCl, well separated from Rd-1, which is a much more acidic protein [18]. This Rd-2 containing fraction was dialysed against 10 mM Tris-HCl and loaded into a DEAE Biogel column (4.5 × 30 cm) equilibrated with the same buffer. The adsorbed proteins were eluted with a Tris-HCl gradient from 10 mM to 300 mM. The first fraction, containing Rd-2, was directly loaded onto an hydroxylapatite column (3 × 25 cm) equilibrated with 100 mM Tris-HCl and eluted with a potassium phosphate gradient from 10 to 350 mM. The fraction containing Rd-2, eluted at ~300 mM potassium phosphate, was applied on a gel filtration (Superdex 30) column, using a High Load system (Pharmacia). The column was run aerobically at 2 ml/min using 50 mM Tris-HCl, 100 mM NaCl pH 7.6 as eluting buffer. After this step, the protein (3.3 mg) was judged to be pure by 12% SDS/PAGE which yielded a single band after Coomassie blue staining.

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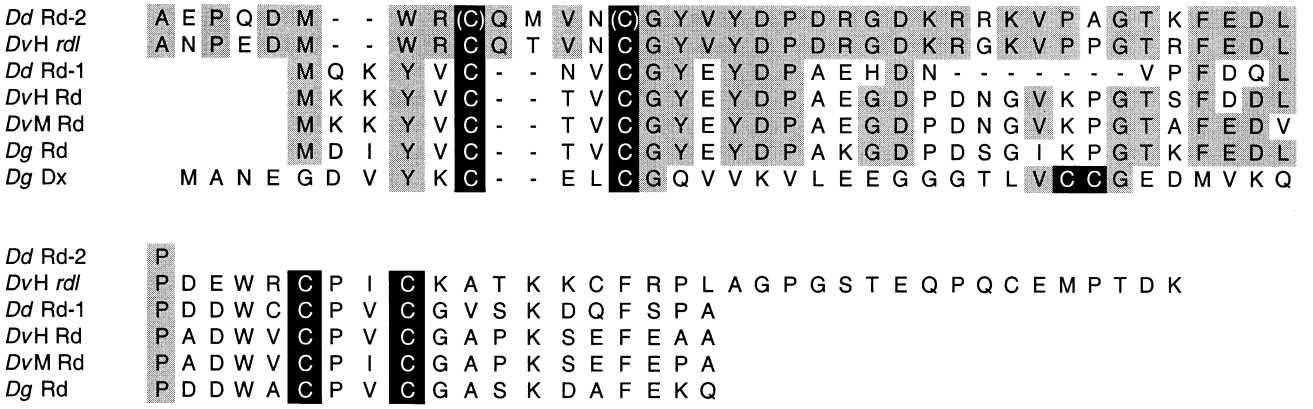

Fig. 1. Sequence alignment of Rd-2 N-terminus with homologous proteins from *Desulfovibrio*. *D. desulfuricans* 27774 rubredoxin 2 (*Dd* Rd-2), *D. vulgaris* Hildenborough RdI (*Dv*H *rdl*), *D. desulfuricans* 27774 rubredoxin 1 (*Dd* Rd-1), *D. vulgaris* Hildenborough rubredoxin (*Dv*H Rd), *D. vulgaris* Myazaki rubredoxin (*Dv*M Rd), *D. gigas* rubredoxin (*Dg* Rd), *D. gigas* desulforedoxin (*Dg* Dx). Black boxes represent the residues involved in iron co-ordination. Grey boxes represent identities between the aligned proteins and *Dd* Rd-2. Residues in parentheses were inferred from the sequence alignment [29].

2.2. Protein quantitation and chemical analysis

The protein N-terminal sequence was obtained using an Applied Biosystem Model 477A sequencer. Amino acid sequence comparisons were performed at the NCBI using the BLAST network service. The protein concentration was determined by the microbiuret method [19]. The iron content was determined chemically by the 2,4,6-tripyridyl-1,3,5-triazine method [20].

2.3. Spectroscopic methods

Room temperature ultraviolet/visible spectra were recorded on a Beckman DU-70 spectrometer. EPR spectra were recorded on a Bruker ESP 380 spectrometer, equipped with an ESR 900 continuous-flow helium cryostat.

2.4. Redox titrations

Visible redox titrations were performed on a Shimadzu spectrometer (UV 265) equipped with a cell stirring system. Rubredoxin 2 (20 μM) was titrated anaerobically in 20 mM Tris-HCl pH 7.6 by stepwise addition of buffered sodium dithionite as in [4], but with further addition of the following redox mediators (0.25 μM each): 1,2-naphthoquinone ($E'_o = 180$ mV), trimethylhydroquinone ($E'_o = 115$ mV), 1,4-naphthoquinone ($E'_o = 60$ mV). The reduction potentials are quoted versus the standard hydrogen electrode.

3. Results and discussion

3.1. Biochemical characterisation

The new rubredoxin from *D. desulfuricans*, named Rd-2, was purified to homogeneity and its molecular mass, as determined by 12% SDS/PAGE, is 8.5 kDa. Gel filtration showed that the protein is a monomer in solution. Chemical analysis revealed the presence of one iron atom per molecule of protein. The N-terminal sequence (Fig. 1) is highly homologous (~75% identity) to the deduced sequence of the *rdl* gene from *D. vulgaris* Hildenborough [21]; in particular, the unusual spacing of the first two cysteine residues, cys-X_4-cys, is also observed, differing from the cys-X_2-cys motif, conserved among rubredoxins in general and also in those from *Desulfovibrio*. These results strongly suggest that *D. desulfuricans* Rd-2 and the *rdl* gene product from *D. vulgaris* Hildenborough are analogous new proteins. Since so far this motif has not been found in any other proteins deposited in the data banks, these two proteins are the first members of a new family of rubredoxins. It is remarkable that, in spite of the different arrangement of the cysteine residues, the overall amino acid homology between these two new rubredoxins and the others is high. In particular the aromatic residues before the first cysteine (a tryptophan in Rd-2 and Rdl, a tyrosine in the other Rds) and two residues after the second cysteine (a tyrosine) are conserved (see [1] for a detailed discussion on these features). Identities ranging from 60 to 95% are found among *Desulfovibrio* rubredoxins, while very little homology is observed with Dx (Fig. 1). This, together with the unusual -cys-cys- motif present, suggests that Dx is distantly related to rubredoxins and is the so far unique member of another sub-family of the small mononuclear non-haem iron proteins, with a sulphur tetrahedral coordination.

3.2. Spectroscopic characterisation

The electronic absorption spectrum of *D. desulfuricans* Rd-2 (Fig. 2, trace a) is similar to those of the other rubredoxins, as expected for an iron centre with the same type of co-ordination to four cysteine residues. Main bands at 350 nm (shoulder at ~380 nm), 480 nm and 575 nm are observed. The main difference towards other rubredoxins, and in particular towards *D. desulfuricans* Rd-1 (Fig. 2, trace b), lies in the rela-

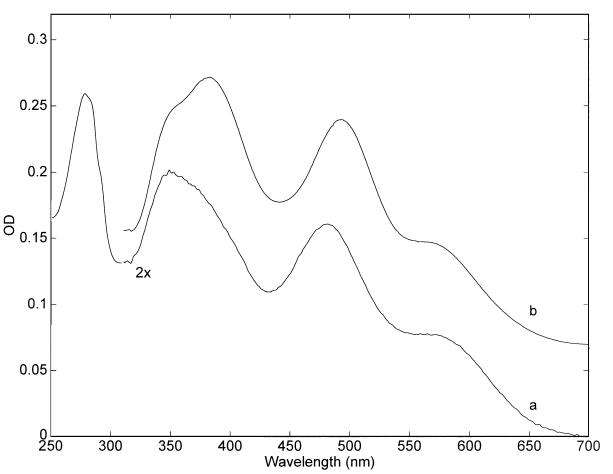

Fig. 2. UV-visible spectra of *D. desulfuricans* native rubredoxins. Trace a: rubredoxin 2; trace b: rubredoxin 1 (offset for clarity). Proteins in 50 mM Tris-HCl buffer pH 7.6.

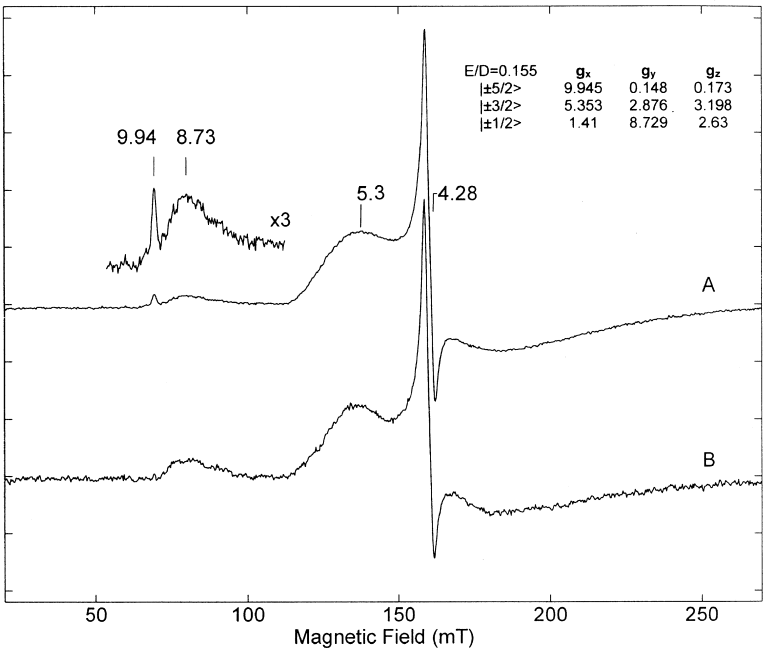

Fig. 3. EPR spectra of *D. desulfuricans* Rd-2. Trace a: Native Rd-2, 4.6 K; trace b: native Rd-2, 20 K. Microwave power: 2.4 mW; microwave frequency, 9.64 GHz; modulation amplitude 0.9 mT. Inset, g values expected for the indicated rhombicity (E/D), yielding effective g values closer to the experimentally observed ones, calculated using the program Rhombo [29].

tive intensity of the 350 and 380 nm bands and in the fact that the 480 nm band is ~10 nm blue shifted. These features are identical to those reported for recombinant *D. vulgaris* Rdl [21]. According to the assignment of the electronic bands to sulphur→metal charge transfer transitions [22], these differences result mainly from the $S \rightarrow d_{xy}$ transitions, indicating a slightly altered geometry at the iron centre. Nevertheless, these differences are less pronounced than in desulforedoxin which, in spite of a similar geometric structure, shows a much more distinct electronic spectrum.

The EPR spectrum also reflects a distinct iron environment, being drastically different from those of regular rubredoxins (Fig. 3). The spectrum shows resonances at $g = 9.94$, 8.73 and ~5. These features can be ascribed to a ferric high-spin site with a rhombic distortion of ~0.155, which yields effective g values of 9.94, 5.35 and 8.73 for the $|\pm 5/2\rangle$, $|\pm 3/2\rangle$ and the $|\pm 1/2\rangle$ doublets, respectively (Fig. 3, inset). Clearly, as mentioned before [16], the EPR of high-spin ferric iron centres, even for the same type of co-ordination and geometry, can be quite diverse and not directly correlated with the nature of the site in terms of coordination number or ligands. Since at 20 K the relative amplitude of the resonance of the $|\pm 1/2\rangle$ doublet increases in relation to the other two (Fig. 3, line B), the $|\pm 5/2\rangle$ doublet is the ground state and the zero field splitting (D) is negative, also in contrast with the other rubredoxins, for which it is positive. The resonance at $g = 4.28$, characteristic of a high-spin ferric centre with $E/D = 0.33$, is assigned to adventitiously bound iron.

3.3. Redox properties

The reduction potential of *D. desulfuricans* Rd-2 was determined by a visible redox titration. The data (Fig. 4) show a single redox transition, with a reduction potential of 25 ± 5 mV ($n = 1$), at pH 7.6. Kinetic assays demonstrated that Rd-2 is reduced by NADH in the presence of *D. desulfuricans* crude extract, which points to the presence of a NADH:Rd-2 oxidoreductase. In fact, several years ago, a flavoprotein with NADH oxidoreductase activity was purified from *D. desulfuricans* [9]. This protein, which was capable of slowly reducing rubrerythrin, but not Rd-1, may be the actual electron donor to Rd-2. The ability of *D. gigas* NADH:rubredoxin oxidoreductase (NRO) to reduce the new *D. desulfuricans* rubredoxin was also investigated. In the presence of NADH, Rd-2 was shown to be only very slowly reduced, as is the case of *D. desulfuricans* Rd-1 [23].

4. Conclusion

D. desulfuricans has been found to contain two types of

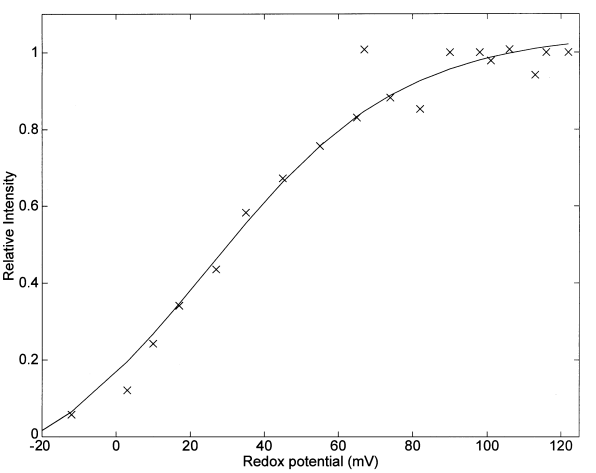

Fig. 4. Visible redox titration of Rd-2. Titration curve of Rd-2, following the absorbance at 480 nm. The line corresponds to a Nernst equation, with $E° = 25$ mV, $n = 1$.

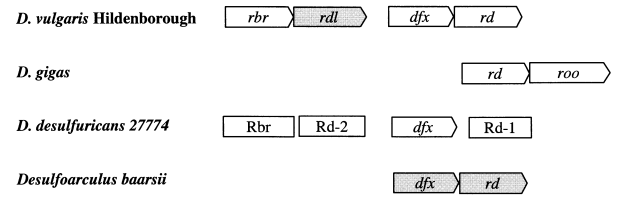

Fig. 5. Comparison of protein and genomic data on *Desulfovibrio* rubredoxins. *Rbr*, Rubrerythrin; Rdl, rubredoxin-like protein (now called Rd-2); *Dfx*, desulfoferrodoxin; *Rd*, rubredoxin; *Roo*, rubredoxin-oxygen oxidoreductase. Arrowhead boxes: gene and protein data available; grey arrowhead boxes: only genetic data available; white boxes: only protein data available. Adjacent arrowhead boxes indicate gene arrangement in an operon.

rubredoxin, which are now designated Rd-1 [24] and Rd-2, described in this paper. A similar situation seems to occur in *D. vulgaris*, although in this organism it is yet not known whether Rd-2 (the *rdl* gene product) is expressed. Thus, *D. desulfuricans* Rd-2 is the second member of a rubredoxin subfamily, differing at the binding motif of the iron site. Protein and genomic data bank analyses reveal that so far only these two proteins contain this feature. Since rubrerythrin is an abundant protein in *D. vulgaris* [10], the gene encoding it is in the same operon as Rdl and both genes are co-transcribed [21], one might expect to find the latter in equivalent amounts as rubrerythrin. It may be that Rd-2 is not stable under the isolation conditions applied previously and that the anaerobic purification conditions now used have been a determining factor in isolating it from wild type cells, or that the translation efficiencies for Rd-2 in *D. vulgaris* and *D. desulfuricans* are quite different. Nevertheless, once purified the *D. desulfuricans* protein is stable.

Although rubrerythrin is also present in *D. desulfuricans*, at present it is not known if, as in *D. vulgaris*, Rd-2 and Rbr are in the same operon. A comparison between the available protein and the genomic data of sulphate-reducing bacteria and archaea [28] shows that type 1 rubredoxins may have diverse gene localisations, which may indicate diverse physiological functions (Fig. 5). In *D. gigas*, Rd is located in the same operon as rubredoxin:oxygen oxidoreductase (Roo), with which has been shown to interact [4]. By the contrary, in *D. vulgaris* [25], *Desulfoarculus baarsii* [26] and *D. desulfuricans* [27], type 1 rubredoxins are located in operons containing Dfx. For that reason Dfx has been designated rubredoxin oxidoreductase, a function which, however, has not been yet determined. In *D. vulgaris* Rbr and Rd-2 are part of a gene cluster which also comprises a gene encoding a putative Fur-(ferric uptake regulatory protein) like protein [21]; such an arrangement is not found in the sulphate-reducing archaeon *Archaeoglobus fulgidus* [28], in spite of the fact that its genome contains four genes encoding proteins with strong similarities to the *Desulfovibrio* rubrerythrins.

The physiological role of Rd-1 and Rd-2 from *D. desulfuricans* remains to be elucidated. With the present data available, it is tempting to speculate that the real electron acceptor for the flavoprotein with NADH oxidoreductase activity [9] is in fact the newly discovered Rd-2. Clearly, it is essential to gather physiological evidence to assign a function to a protein. Studies are in progress to elucidate this issue.

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